CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Jan. 2001, p. 181–186 Vol. 8, No. 1
1071-412X/01/$04.00+0 DOI: 10.1128/CDLI.8.1.181–186.2001

Determination of *Helicobacter pylori* Virulence by Simple Gene Analysis of the *cag* Pathogenicity Island

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Received 10 May 2000/Returned for modification 4 August 2000/Accepted 20 September 2000

Nucleic acid amplification was performed for five loci in the *cag* pathogenicity island (PAI) of *Helicobacter pylori* (comprising *cagA*, the *cagA* promoter region, *cagE*, *cagT*, and the left end of *cag*II [LEC]), and gastric inflammation in patients was evaluated. Of 204 *H. pylori* isolates from Japanese patients (53 with peptic ulcer, 55 with gastric cancer, and 96 with chronic gastritis), 197 (96.6%) were positive for all five loci. Two isolates (1%) were negative for all five loci, and five isolates (2.4%) were positive for only *cagA* and LEC. These latter seven isolates were all from patients with mild chronic gastritis. Neutrophil infiltration in gastric mucosa was significantly milder in patients infected with partially or totally deleted-PAI strains than in those with intact-PAI strains. The *cagE* gene was a more accurate marker of an intact *cag* PAI than the *cagA* gene, and *cagE* seemed to be more useful in discriminating between *H. pylori* strains causing different rates of disease progression.

Helicobacter pylori is a gram-negative, spiral-shaped, microaerophilic bacterium that infects human gastric mucosa and is recognized as a major cause of chronic active gastritis, peptic ulcer disease, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma (10, 16, 17, 18, 20, 23, 33). Although the pathogenesis of *H. pylori* infection is not well understood, there are several putative virulence factors that may contribute to mucosal damage by *H. pylori* infection.

The cytotoxin-associated-gene (*cag*) pathogenicity island (PAI) is an approximately 40-kb cluster of genes in the *H. pylori* chromosome (4, 29) and is divided into two regions, *cag*I and *cag*II. There are at least 14 and 16 open reading frames (ORFs) in *cag*I and *cag*II, respectively. Some of the ORFs in the *cag* PAI are believed to encode proteins which have similarities to other bacterial secretion systems, such as the *Bordetella pertussis* toxin secretion system (4).

The *cag* PAI is considered to be one of the major virulence factors of *H. pylori* (4). Extensive studies of the *cagA* gene, located in the most downstream portion of the *cag* PAI, have indicated that the CagA protein is associated with peptic ulcer disease, gastric cancer, and mucosa-associated lymphoid tissue lymphoma in the stomach (3, 5, 11, 13, 14, 22, 24, 25, 27, 30, 32). Blaser et al. (3) revealed that CagA antibodies were more frequently detected in *H. pylori*-infected patients with gastric cancer than in those without gastric cancer (odds ratio, 1.9). Furthermore, Parsonnet et al. (24) showed that subjects infected with *H. pylori* who had CagA antibodies were more likely to develop gastric cancer as compared with uninfected subjects (odds ratio, 5.8), while *H. pylori*-infected subjects without CagA antibodies were at only slightly and not significantly increased risk for cancer (odds ratio, 2.2). Thus, the *cagA* gene is conventionally used as a marker of pathogenic strains. However, several studies suggest that the *cagA* gene cannot be used as a suitable marker for *cag* PAI-associated virulence for the following reasons: (i) although *cag* PAI-intact *H. pylori* strains are shown to induce interleukin-8 secretion from gastric epithelial cells (1, 4, 6, 7, 15, 26), an inactivation of some *cag* PAI genes such as *cagE* but not *cagA* causes a marked reduction in the ability of *H. pylori* to induce interleukin-8 induction (1, 4, 8, 21, 31); (ii) we have previously shown that some Japanese strains obtained from patients with nonulcer dyspepsia lack most of the *cag* PAI genes, including the promoter region of the *cagA* gene, despite the presence of the *cagA* gene itself, indicating that the presence of the *cagA* gene does not always signify the presence of an intact *cag* PAI and an ability to produce CagA protein (15). Although recent studies have revealed that the CagA protein is translocated into the host cells and tyrosine phosphorylated, the precise role of the CagA protein in *H. pylori* pathogenesis is still unknown (2, 19, 28). These findings may suggest that a gene other than *cagA* can be used as a marker for *cag* PAI-associated virulence.

By using our recombinant CagA protein and antibodies, we

TABLE 1. Sequences and locations of oligonucleotide primers

Primer	Primer sequence	Location[a]
cagA-F1	5′-AACAGGACAAGTAGCTAGCC-3′	2,700–2,719*
cagA-F2	5′-GATAACAGGCAAGCTTTTGA-3′	157–176*
cagA-R1	5′-TATTAATGCGTGTGTGGCTG-3′	3,400–3,381*
cagA-R2	5′-CTGCAAAAGATTGTTTGGCAGA-3′	505–484*
cagE-F1	5′-GCGATTGTTATTGTGCTTGTAG-3′	16,891–16,870†
cagE-R1	5′-GAAGTGGTTAAAAAATCAATGCCCC-3′	16,563–16,587†
cagT-F1	5′-CCATGTTTATACGCCTGTGT-3′	442–461†
cagT-R1	5′-CATCACCACACCCTTTTGAT-3′	723–742†
cagAP-F1	5′-GTGGGTAAAAATGTGAATCG-3′	18,738–18,757†
cagAP-F2	5′-CTACTTGTCCCAACCATTTT-3′	18,495–18,514†
LEC-F1	5′-ACATTTTGGCTAAATAAACGCTG-3′	3,920–3,942‡
LEC-F2	5′-ATAGCGTTTTGTGCATAGAA-3′	3,856–3,875‡
LEC-R1	5′-TCTCCATGTTGCCATTATGCT-3′	4,303–4,283‡
LEC-R2	5′-ATCTTTAGTCTCTTTAGCTT-3′	4,732–4,713‡

[a] GenBank accession no.: *, AF001357; †, U60176; ‡, AC000108.

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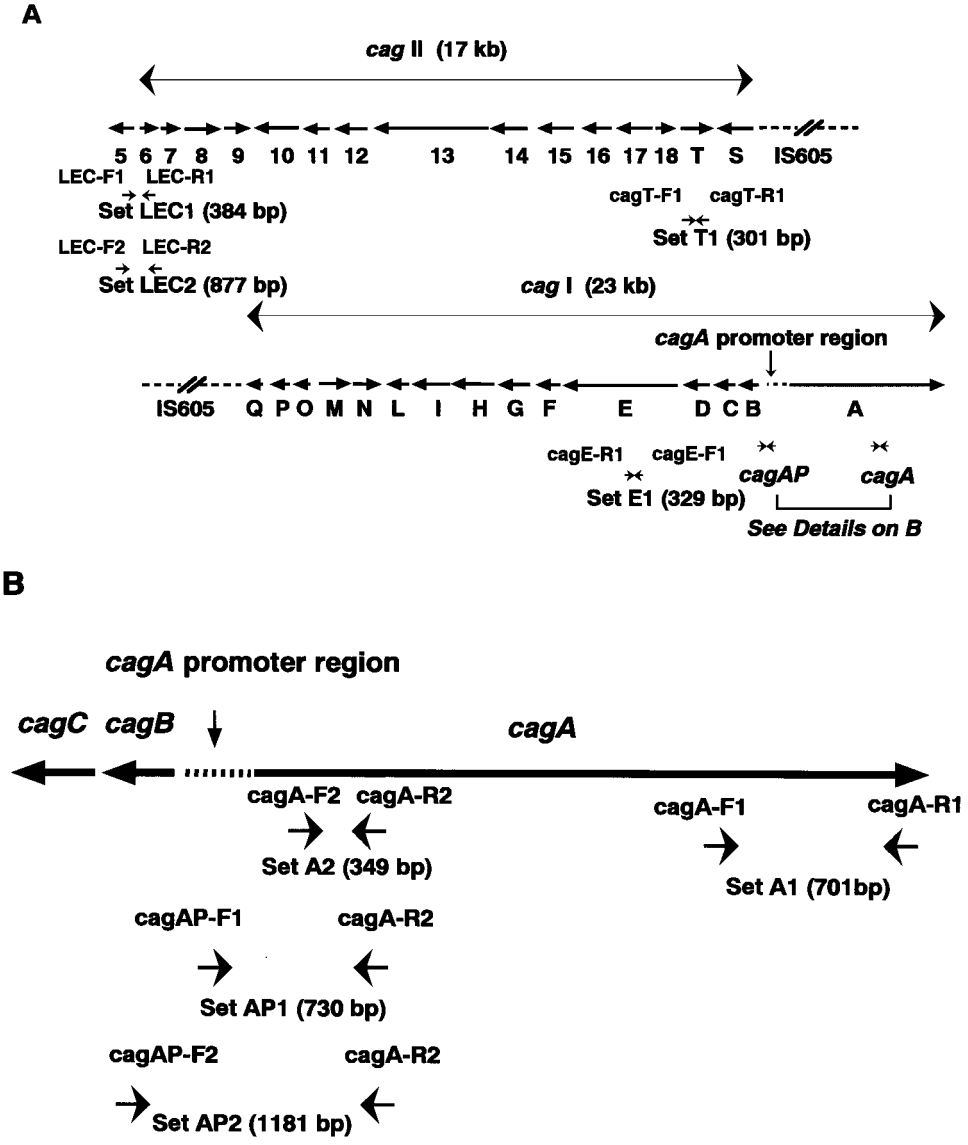

FIG. 1. Structure of *cag* pathogenicity island and locations of PCR primers. (A) Locations of PCR primers for *cagE*, *cagT*, and the LEC. Two sets of primers were used to detect the *cagA* gene: primer set A1 (cagA-F1 and cagA-R1) and set A2 (cagA-F2 and cagA-R2). For detection of the promoter region of *cagA*, primer sets AP1 (cagAP-F1 and cagA-R2) and AP2 (cagAP-F2 and cagA-R2) were used, and for the left end of *cag*II, primer sets LEC1 (LEC-F1 and LEC-R1) and LEC2 (LEC-F2 and LEC-R2) were used. The expected lengths of PCR products using each primer set are shown. The sequence and location of each primer are shown in Table 1. (B) Locations of PCR primers for the *cagA* gene and its promoter region. The expected lengths of PCR products using each primer set are also shown.

previously showed a high prevalence of CagA-producing *H. pylori* strains in Japan (13, 14). Furthermore, by using Southern blot hybridization with DNA probes obtained by cloning 15 different ORFs in *cag* PAI, we clarified the details of DNA structure in the entire *cag* PAI (15) and found that Japanese strains deficient in the *cagA* gene lacked most of the *cag* PAI genes, including the *cagE* gene and the promoter region of the *cagA* gene. However, it is troublesome to check all *cag* PAI genes by Southern blotting.

Thus, in the present study, we attempted to establish a simple and practical method for determining the structure of *cag* PAI and consequently discriminating between Japanese *H. pylori* strains causing different rates of disease progression.

A total of 204 *H. pylori* isolates was obtained from *H. pylori*-infected adults who had undergone upper gastrointestinal endoscopy at Tokyo University Hospital. The patients consisted of 145 men and 59 women with a mean age of 58.5 years (ranging from 22 to 85 years). Patient endoscopic findings were as follows: gastric cancer in 55 patients, gastric ulcer in 22, duodenal ulcer in 17, both gastric and duodenal ulcers in 14, and chronic gastritis in 96.

Gastric biopsy specimens were cultured on Columbia agar with 5% (vol/vol) horse blood and Dent antibiotic supplement (Oxoid, Basingstoke, United Kingdom) at 37°C for 5 days under microaerobic conditions (CampyPak System; BBL, Cockeysville, Md.). Organisms were identified as *H. pylori* by colony morphology and gram staining, as well as positive activity for urease, catalase, and oxidase. The isolates were stored at

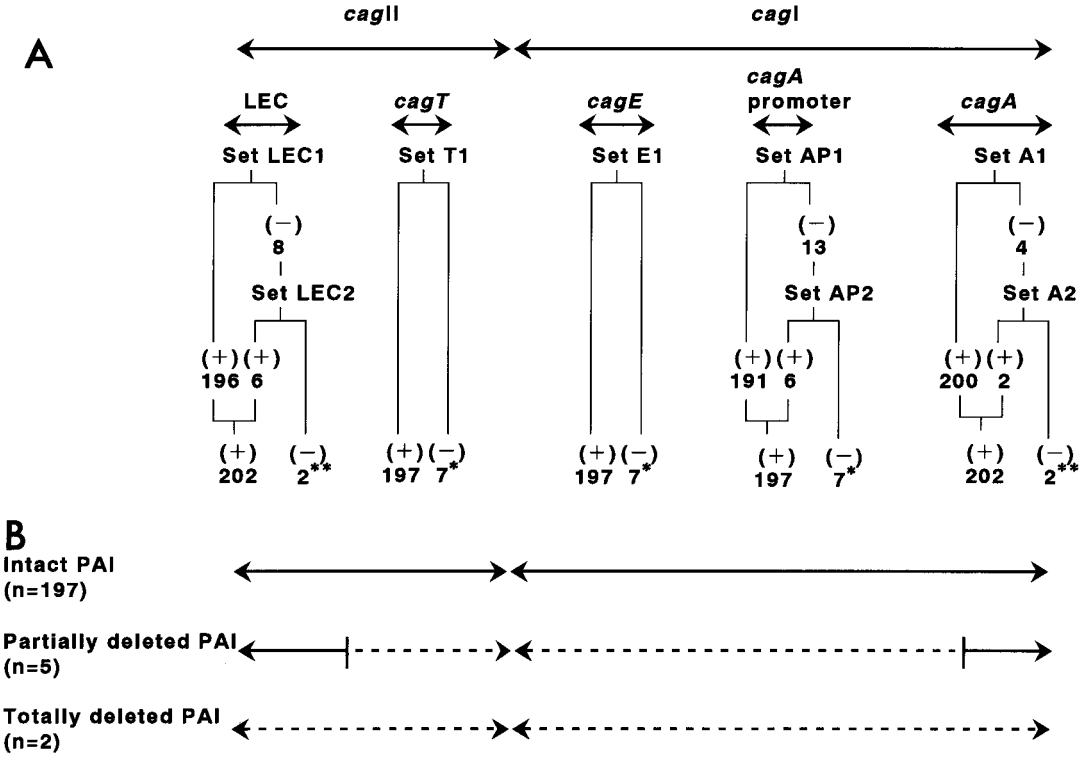

FIG. 2. Results of *cag* PAI gene PCR in 204 Japanese *H. pylori* isolates. (A) Positivity of *cag* PAI gene PCR by each primer set: *, seven *cagA* promoter PCR-negative isolates also negative for both *cagE* and *cagT* PCR; **, two isolates negative for LEC PCR and also negative for *cagA* PCR. (B) Types of *cag* PAI structures determined in this study.

−80°C in brucella broth with 5% (vol/vol) fetal bovine serum containing 16% (vol/vol) glycerol. DNA was prepared as described previously (15).

Five different loci allowing for structure screening of *cag* PAI were selected on the basis of our previous Southern blot analysis (15). The previous study revealed that when the ORFs of *cag* PAI were deleted, the deletions started from the region between *cagA* and the *cagA* promoter region through *cagQ* (*cag*I) and continued from *cagS* through *cag-13* or *cag-8* (*cag*II) (15). Thus, *cagA*, the *cagA* promoter region, and *cagE* were selected to represent *cag*I, and *cagT* and the left end of *cag*II (LEC) were selected to represent *cag*II. Therefore, overall five loci were selected.

Pairs of oligonucleotide primers were used to detect the presence of the *cag* PAI genes *cagA*, the *cagA* promoter region, *cagE*, *cagT*, and the LEC, containing both inside and outside genes of *cag* PAI, and these primer pairs were designed on the basis of published sequences reported by Censini et al. (GenBank accession number, U60176), Akopyants et al. (GenBank accession number, AC000108), Tomb et al. (GenBank accession number, AE000511), and ourselves (GenBank accession number, AF001357) (Table 1; Fig. 1). As shown in Fig. 1A and B, two sets of primers were used to detect the *cagA* gene (sets A1 and A2), the *cagA* promoter region (sets AP1 and AP2), and the LEC (sets LEC1 and LEC2). To detect *cagE* and *cagT*, one set of primers was used for each gene, set E1 and set T1, respectively. *H. pylori* strains ATCC 43526 and 43579, which have been determined to have the entire *cag* PAI (15), were used as positive controls for each PCR. Since eight *cagA* gene-negative strains from Western countries, including Tx30a, kindly provided by J. C. Atherton (Nottingham University, United Kingdom), were determined to lack the entire *cag* PAI (15), these were used as negative controls. The genomic DNAs from other bacterial species—*Escherichia coli*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Campylobacter fetus*, *Campylobacter jejuni*, *Klebsiella pneumoniae*, *Citrobacter freundii*, and *Enterobacter aerogenes*—were tested using each primer set to assess the specificity of each PCR.

For histological analysis, biopsy specimens from corpus and antrum were embedded in paraffin, stained with hematoxylin and eosin, and examined by two pathologists blinded to the patient's clinical diagnosis or characteristics of the *H. pylori* strain. The presence of chronic active gastritis was determined by scoring the following parameters on the basis of the updated Sydney System (9): density of inflammatory infiltration (0 to 3)

TABLE 2. Relationship between presence of *cag* PAI genes and clinical diagnosis

Diagnosis (total patients)	Patients with *cag* type infection:		
	Intact PAI	Partially deleted PAI	Totally deleted PAI
Peptic ulcer disease (53)	53	0	0
Gastric cancer (55)	55	0	0
Chronic gastritis (96)	89	5	2
Total (204)	197	5	2

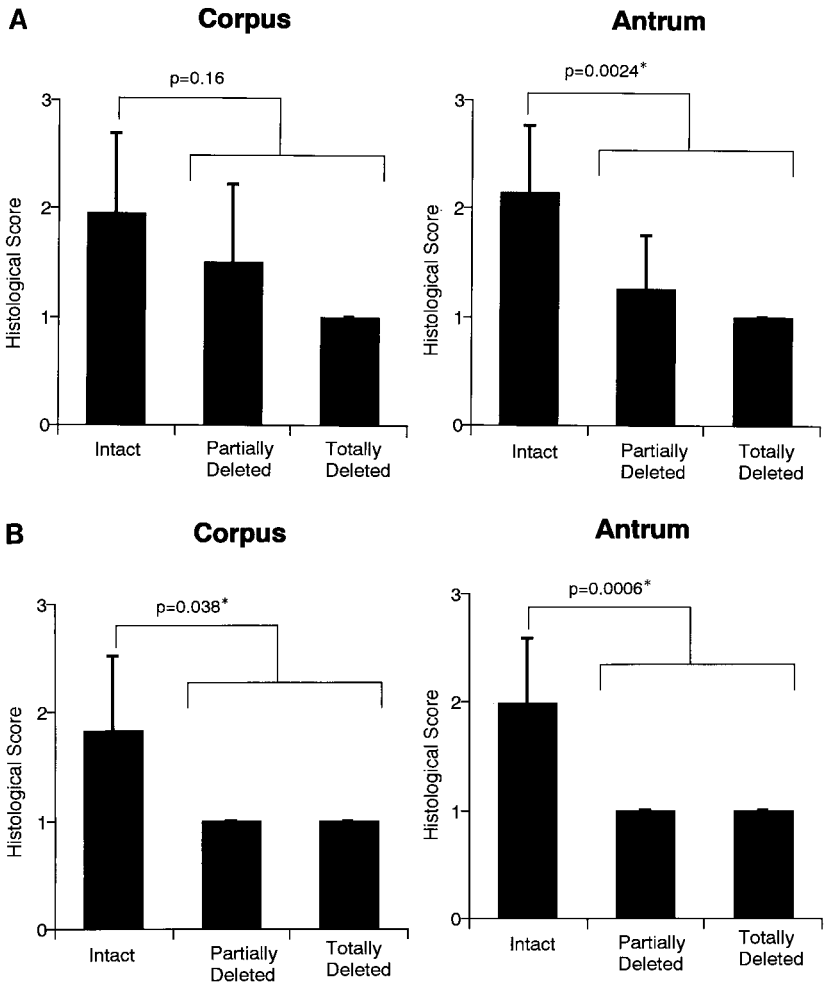

FIG. 3. Relationship between gastric inflammation and the presence of *cag* PAI genes of infected *H. pylori* strains. A total of 64 patients (59 infected with strains with intact type, 4 with partially deleted type, and 1 with totally deleted type) were assessed for (A) inflammatory infiltration and (B) neutrophil infiltration in corpus and antral mucosa. Each valuable was scored on a four-point histological scale (0, none; 1, mild; 2, moderate; and 3, severe). *, statistically significant by Mann-Whitney U test.

and density of neutrophil infiltration (0 to 3). For each parameter, 0 is none, 1 is mild, 2 is moderate, and 3 is severe.

PCR amplification specificity for *cagA*, the *cagA* promoter region, *cagE*, *cagT*, and the LEC was assessed by testing *H. pylori* strains ATCC 43526 and 43579 and eight *cagA* gene-negative strains from Western countries, as well as 10 other bacterial species. Only *H. pylori* strains ATCC 43526 and 43579 were positive for PCR amplification of all five loci. Eight *cagA* gene-negative Western strains and the other bacterial species tested were all negative for PCR of all five loci. Thus, the specificity of PCR for each PAI locus was 100%.

To assess the sensitivity of PCR for each locus, excluding the *cagA* promoter region, PCR was performed with 30 *H. pylori* isolates from Japanese patients whose *cag* PAI gene status was determined by Southern blot analysis in our previous study (15). PCR results for *cagA* (primer sets A1 and A2), *cagE* (primer set E1), *cagT* (primer set E1), and the LEC (primer sets LEC1 and LEC2) were completely consistent with those of previous Southern blot analyses (15). Thus, if at least two sets of primers were used, the specificity of PCR for each PAI locus was 100%.

As shown in Fig. 2 and Table 2, 202 out of 204 (99.0%) isolates were positive for *cagA* and LEC, and 197 out of 204 (96.6%) isolates were positive for the *cagA* promoter region, *cagE*, and *cagT*. Since two *cagA*-negative strains were also negative for all other genes tested and the remaining five out of seven *cagA* promoter-negative strains were negative for *cagE* and *cagT*, the *cag* PAI genes present in Japanese *H. pylori* isolates were divided into three types; intact-PAI, partially deleted-PAI, and totally deleted-PAI genes (Fig. 2).

Recently, Jenk et al. reported that the presence of the entire *cag* PAI is highly related to duodenal ulcers but that the clinical outcome of *H. pylori* infection is not reliably predicted by analyzing several genes of the *cag* PAI, including *cagA*, *cagE*, and *cagT* (12). In their study, the presence of *cagE* was completely consistent with that of *cagA* but not *cagT*. In contrast, our study revealed consistency in the presence of *cagE* with *cagT* but not *cagA*, indicating that the strain diversity may exist

in relation to *cag* PAI genes among Western countries and Japan.

Strains with partially or totally deleted *cag* PAIs, which lack both *cagE* and *cagT*, were more frequently found in more patients with chronic gastritis only (7 out of 96 patients [7.3%]) than with peptic ulcer disease (0 out of 53; $P = 0.042$) or with gastric cancer (0 out of 55; $P = 0.039$) (Table 2). Furthermore, by assessing inflammation activity in the gastric mucosa of 64 patients (59 infected with intact-PAI-type strains, 4 with partially deleted-PAI-type strains, and 1 with a totally deleted-PAI-type strain), we found no significant differences in inflammatory infiltration of corpus between patients with intact type strains and those with partially or totally deleted type strains. However, inflammatory infiltration in antrum (Fig. 3A) and neutrophil infiltration in corpus and antrum (Fig. 3B) were significantly milder in patients with partially or totally deleted type strains than in those with intact type strains. These findings suggest that the strains with partially or totally deleted PAI may have weaker ability to cause disease progression than those with intact PAI.

In the present study, the partially or totally deleted type strains in Japan lacked *cagE*, *cagT*, and the *cagA* gene promoter region, regardless of the presence of the *cagA* gene itself. Therefore, they could be discriminated from intact type strains by detection of *cagE*, *cagT*, or the *cagA* gene promoter region but not by detection of the *cagA* gene itself. Although the *cagA* gene is conventionally used as a marker for virulence, especially with the PCR amplification method, our results indicate that not the *cagA* gene itself but the promoter region of the *cagA* gene could be a better marker. However, due to the diversity of the *cagA* gene promoter region sequences, designing specific primers to detect this region may be difficult. Since the *cagE* gene is located near the *cagA* gene promoter region and retained consistently within this region, it seems valid to choose the *cagE* gene as a substitute for the *cagA* gene promoter region. Since the primer sets designed for *cagE* PCR in this study were extremely specific and sensitive, at least for Japanese strains, we conclude that *cagE* PCR can be used as a practical method for screening the status of the *cag* PAI structure, which may be related to disease progression, for a large number of samples in order to test clinical significance or to conduct an epidemiological survey.

In conclusion, the results of the present study indicate that *cagE* is more accurate, as a marker of an intact *cag* PAI, than the *cagA* gene and that it seems to be more useful in discriminating between *H. pylori* strains with different rates of disease progression in Japan. Detection of the *cagE* gene by PCR amplification with specific primers can be used as a simple and practical method for their discrimination.

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